

TAB F

Cannabinoid receptors as novel targets for the treatment of melanoma

Cristina Blázquez,* Arkaitz Carracedo,* Lucía Barrado,[†] Pedro José Real,[‡] José Luis Fernández-Luna,[‡] Guillermo Velasco,* Marcos Malumbres,[†] and Manuel Guzmán*¹

*Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, Madrid, Spain; [†]Cell Division and Cancer Group, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain; and [‡]Unit of Molecular Genetics, Marqués de Valdecilla University Hospital, Santander, Spain

ABSTRACT Melanoma causes the greatest number of skin cancer-related deaths worldwide. Despite intensive research, prevention and early detection are the only effective measures against melanoma, so new therapeutic strategies are necessary for the management of this devastating disease. Here, we evaluated the efficacy of cannabinoid receptor agonists, a new family of potential antitumoral compounds, at skin melanoma. Human melanomas and melanoma cell lines express CB₁ and CB₂ cannabinoid receptors. Activation of these receptors decreased growth, proliferation, angiogenesis and metastasis, and increased apoptosis, of melanomas in mice. Cannabinoid antimelanoma activity was independent of the immune status of the animal, could be achieved without overt psychoactive effects and was selective for melanoma cells vs. normal melanocytes. Cannabinoid antiproliferative action on melanoma cells was due, at least in part, to cell cycle arrest at the G1-S transition via inhibition of the pro-survival protein Akt and hypophosphorylation of the pRb retinoblastoma protein tumor suppressor. These findings may contribute to the design of new chemotherapeutic strategies for the management of melanoma.—Blázquez, C., Carracedo, A., Barrado, L., Real, P. J., Fernández-Luna, J. L., Velasco, G., Malumbres, M., and Guzmán, M. Cannabinoid receptors as novel targets for the treatment of melanoma. *FASEB J.* 20, E2199–E2208 (2006)

Key Words: G protein-coupled receptor · antitumoral drug · experimental therapeutics · cell proliferation

MELANOMA CAUSES THE GREATEST number of skin cancer-related deaths both in the USA (1) and worldwide (2). In 2005, an estimated 59,600 Americans were diagnosed with this cancer and 7,800 died of it (1, 3). At present, prevention and early detection are the only effective measures against melanoma. Thus, melanoma basically remains a surgical disease, and excision of thin, biologically early tumors offers the best chance of cure. Despite intensive research, no effective therapies exist for advanced melanoma. Only high-dose IFN α -2b

has a reproducible benefit in stage II and III patients, but owing to its numerous side effects, modest efficacy, and high cost, it is not used worldwide and is inconsistently used in the United States (2, 4, 5). For patients with stage IV melanoma, randomized, controlled trials have shown no significant advantage of any specific drug or combination of drugs. Dacarbazine, the only cytotoxic drug approved by the FDA for the treatment of metastatic melanoma, remains the benchmark, but it produces responses of moderate duration and only in a small fraction of the patients (2, 4, 5). New therapeutic strategies are therefore necessary for the management of melanomas.

Cannabinoid receptors, a family of G protein-coupled receptors that are normally engaged by a family of endogenous ligands—the endocannabinoids anandamide (6) and 2-arachidonoylglycerol (7)—and to which active components of the hemp plant *Cannabis sativa* L. cross-bind, participate in the control of a wide array of biological processes (8, 9). Two different cannabinoid receptors have been cloned from mammalian tissues (8): the CB₁ receptor, mostly expressed in brain, and the CB₂ receptor, mostly expressed in the immune system. Preparations of cannabis have been used in medicine for many centuries, and currently, there is an intense renaissance in the study of the therapeutic effects of cannabinoids focused on the design of potent and selective synthetic cannabinoid agonists and antagonists (8, 9). Cannabinoids have been known to exert palliative effects in cancer patients since the early 1970s. The best established of these effects is the inhibition of chemotherapy-induced nausea and vomiting. Today, capsules of Δ^9 -tetrahydrocannabinol (THC), the major active component of cannabis, and its synthetic analog nabilone are approved by several countries for that purpose (10–12). Other potential palliative effects of cannabinoids in oncology—supported by phase III clinical trials—include appetite

¹ Correspondence: Department of Biochemistry and Molecular Biology I, School of Biology, Complutense University, 28040 Madrid, Spain. E-mail: mgp@bbm1.ucm.es
doi: 10.1096/fj.06-6638fj

stimulation and pain inhibition (11, 12). In addition, cannabinoids are potential antitumoral agents owing to their ability to inhibit the growth and angiogenesis of various types of tumor xenografts in animal models (11, 13–15). Cannabinoids display a fair drug safety profile in both animals and humans and do not produce the generalized cytotoxic effects of conventional chemotherapies (11, 16–18). In this context, we have recently run a pilot clinical trial aimed at investigating the effect of THC administration on the growth of recurrent glioblastoma multiforme (19).

This background prompted us to explore the potential utility of cannabinoids as antitumoral agents against malignant melanoma. Specifically, we studied in melanoma cells 1) the expression of cannabinoid receptors, 2) the growth-inhibiting effect of cannabinoids, and 3) the mechanism of cannabinoid action. Our data show that 1) melanoma cells express functionally active CB₁ and CB₂ cannabinoid receptors, 2) cannabinoid receptor activation inhibits melanoma cell growth *in vitro* and *in vivo*, and 3) cannabinoids act on melanoma cells, at least in part, by arresting the cell cycle at the G1-S transition via inhibition of the prosurvival protein Akt and hypophosphorylation of the pRb retinoblastoma protein tumor suppressor.

MATERIALS AND METHODS

Cannabinoids

JWH-133 was kindly given by John W. Huffman (Department of Chemistry, Clemson University, SC), THC by Alfredo Dupetit (The Health Concept, Richelbach, Germany), and SR141716 and SR144528 by Sanofi-Aventis (Montpellier, France). WIN-55,212-2 was from Sigma (St. Louis, MO) and AM630 was from Tocris (Ellisville, MO). For *in vitro* incubations, cannabinoid agonists and antagonists were directly applied at a final DMSO concentration of 0.1–0.2% (v/v). For *in vivo* experiments, ligands were prepared at 1% (v/v) DMSO in 100 μ l PBS supplemented with 5 mg/ml BSA. No significant influence of the vehicle was observed on any of the parameters determined.

Cell culture

The melanoma cell lines B16 (mouse; kindly given by José C. García-Borrón, Murcia University, Spain), A375 (human) and Mefuso (human; kindly given by Alberto Anel, Zaragoza University, Spain) were routinely maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Twenty four hours before the experiments, cells were transferred to low (0.5%) serum DMEM.

The nontumorigenic melanocytic cell lines melan-c (mouse) and Hermes 2b (human), immortalized by telomerase reverse transcriptase expression, were kindly given by Dorothy C. Bennett (St. George's Hospital Medical School, London, UK) and Lluís Montoliu (National Biotechnology Centre, Madrid, Spain) and were maintained in 10% CO₂ atmosphere. Melan-c cells were cultured in RPMI supplemented with 10% fetal calf serum, 2 mM glutamine, 7.5 μ g/ml extra phenol red and 200 nM PMA (Sigma). Hermes 2b cells were cultured in RPMI supplemented with 10% fetal calf serum, 2 mM glutamine, 7.5 μ g/ml extra phenol red, 200

nM PMA, 200 pM cholera toxin (Sigma), 10 nM endothelin 1 (Sigma) and 10 ng/ml human stem cell factor (Chemicon Temecula, CA). Twenty four hours before the experiments, cells were transferred to their respective low-serum media.

Viability and cell cycle in culture

Cell viability in the cultures was determined by trypan blue exclusion. Cell cycle analysis was performed by flow cytometry determination of nuclear DNA content. Cells were detached with trypsin-EDTA (BioWhittaker, Walkersville, ME), collected by centrifugation, washed once, and incubated (1 h, room temperature) in PBS containing 1% (w/v) BSA, 30% ethanol and 5 μ g/ml Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). Fluorescence intensity was analyzed with a LSR flow cytometer (Becton Dickinson, San Jose, CA). Ten thousand cells were recorded in each determination.

Tumor induction in mice

Tumors were induced in C57BL/6 or nude mice by subcutaneous (s.c.) flank inoculation of 1×10^5 B16 melanoma cells. When tumors had reached an average volume of 300 mm³ (range, 200–400 mm³), animals were assigned randomly to the various groups and injected peritumorally (at approx. 2 mm from the tumor) for 8 days with vehicle or cannabinoid (either WIN-55,212-2 or JWH-133 at 50 μ g/day, daily). Tumors were measured with external caliper, and volume was calculated as $(4\pi/3) \times (\text{width}/2)^2 \times (\text{length}/2)$.

Tumor proliferation, apoptosis and angiogenesis in mice

For proliferation assays, mice received an intraperitoneal (i.p.) injection of 5-bromo-2'-deoxyuridine (bromodeoxyuridine; 120 mg/kg body wt) (Boehringer, Mannheim, Germany) 6 h before tumor harvesting. Detection of bromodeoxyuridine (BrdU)-positive cells was performed using an anti-bromodeoxyuridine mouse monoclonal antibody (mAb) (Abcam, Cambridge, UK), as described previously (20). Apoptosis was determined with a TUNEL kit (Boehringer, Mannheim, Germany), according to manufacturer's instructions. Immunodetection of blood vessels was performed with an anti-human CD31 mAb (1:400; Cymbus Biotechnology, Hampshire, UK) as described (21). In all cases, sections were mounted with Mowiol mounting medium (Merck, Darmstadt, Germany) containing YOYO-1 iodide (1:1000; Molecular Probes, Leyden, The Netherlands) to stain cell nuclei, and fluorescence images were acquired using an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany). Morphometric analysis of the vasculature was performed with MetaMorph-Offline software, version 6.2 (Universal Imaging, Downingtown, PA). Inclusive fluorescence thresholds were set at 105 (low) and 255 (high). The number of blood vessels per area unit and the blood vessel sectional area were determined in 5–10 fields of 4–6 sections per tumor.

Generation of metastatic nodules in mice

B16 melanoma cells (5×10^5) were injected into the left paw of C57BL/6 or nude mice. Animals were injected i.p. for 21 days (C57BL/6 mice) or 12 days (nude mice) with vehicle or WIN-55,212-2 (50 μ g/day, every 3 days). Lungs and livers were dissected and fixed in Bouin's solution, and the number of metastatic nodules was counted.

Cannabinoid receptor expression

Reverse transcriptase-polymerase chain reaction

RNA was isolated with the RNeasy Protect kit (Qiagen, Venlo, The Netherlands) including a DNase digestion step using the RNase-free DNase kit (Qiagen). cDNA was obtained with Transcriptor (Roche). Primers were used to amplify mRNA for human CB₁ [5'-CGTGGGACGCTGTCTTCTCA-3' and 5'-CATGGGGGCTTGGTCTGG-3' (408-bp product)], mouse CB₁ [5'-TCTCTGGAAGGCTACAG-3' and 5'-TGTCTGTGGACA-CAGACATG-3' (509-bp product)], human CB₂ [5'-GGCGGG-AAGCCCTCATACC-3' and 5'-CCTCATTCGGGGCATCTCTG-3' (522-bp product)], mouse CB₂ [5'-CGGGAAGAAGAGGATG-CGAATGAAT-3' and CTGCTGAGCGGCTCGAGAAC (479-bp product)] and human/mouse glyceraldehyde 3-phosphate dehydrogenase [5'-GGGAAGCTGACTGGCATGGCCTTCC-3' and 5'-CATGTGGGCCATGAGGCTCCACCA-3' (322-bp product)]. Polymerase chain reaction (PCR) reactions were performed using the following parameters: 95°C for 5 min, 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min (40 cycles), followed by a final extension step at 72°C for 5 min.

Western blot analysis

Particulate cell or tissue fractions were subjected to SDS-PAGE, and proteins were transferred from the gels onto PVDF membranes. The blots were incubated with polyclonal antibodies raised against residues 1–14 of the human/mouse CB₁ receptor (1:10000; kindly given by Allyn Howlett, NC Central University, Durham, NC) or residues 1–99 of the human CB₂ receptor (1:1000; Affinity Bioreagents, Golden, CO) as described (20). Antigen preabsorption experiments were performed by preincubating (37°C, 1 h) 1 µl (=0.5 µg) of anti-CB₁ or anti-CB₂ antibody (Ab) and 100 µl PBS with or without 20 µl (=20 µg) of the corresponding immune peptide. α-Tubulin (1:4000, Sigma) was used as a loading control. In all cases, samples were subjected to luminography with an enhanced chemiluminescence (ECL) detection kit (Amersham Life Sciences, Arlington Heights, IL).

Confocal microscopy

Formalin-fixed, paraffin-embedded sections of 61 human cutaneous melanomas were obtained from the files of the Tumor Bank Network of Centro Nacional de Investigaciones Oncológicas (CNIO, Madrid, Spain). Sections (5 µm) were stained with anti-CB₁ Ab (1:500; Affinity Bioreagents, Golden, CO) or anti-CB₂ Ab (1:500; Affinity Bioreagents), as described previously (22). Slices were further incubated (1 h, room temperature, darkness) with a secondary anti-rabbit Ab-Alexa Fluor 594 (1:400; Molecular Probes, Leyden, The Netherlands). Antigen preabsorption experiments were performed by preincubating the anti-CB₁ or anti-CB₂ Ab with or without the corresponding immune peptide (Affinity Bioreagents), as described above. Control immunostainings using the secondary Ab in the absence of the primary Ab were also performed. Confocal fluorescence images were acquired using Laser Sharp 2000 software (Bio-Rad, Hercules, CA) and a Confocal Radiance 2000 coupled to Axiocvert S100 TV microscope (Carl Zeiss, Oberkochen, Germany).

Expression of signaling proteins

Western blot analysis

Samples were subjected to Western blot analysis using antibodies against phospho-pRb (S807/811) (1:1000; Cell Signaling

Technology, Beverly, MA), pRb (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), p21 (1:200; Santa Cruz Biotechnology), phospho-p27 (T157) (1:2000; R&D Systems, Minneapolis, MN), p27 (1:1000; Santa Cruz Biotechnology), cyclin D1 (1:200; Santa Cruz Biotechnology), CDK4 (1:1000; Pharmingen International, Bormen, Belgium), phospho-CDK2/3 (T160) (1:10000; Santa Cruz Biotechnology), phospho-p53 (S15) (1:1000; Cell Signaling Technology), p53 (1:200; Calbiochem, La Jolla, CA), phospho-Akt (S473) (1:1000; Cell Signaling Technology), Akt (1:1000; Cell Signaling Technology), phospho-ERK (T202/Y204) (1:1000; Santa Cruz Biotechnology), ERK (1:1000; Cell Signaling Technology), phospho-p38 MAPK (T180/Y182) (1:1000; Cell Signaling Technology), p38 MAPK (1:1000; Cell Signaling Technology), phospho-c-Jun NH2-terminal kinase (T183/Y185) (1:1000; Promega, Madison, WI) and c-Jun NH2-terminal kinase (JNK) (1:1000; Cell Signaling Technology). Densitometric analysis of the blots was performed with Multianalyst software (Bio-Rad Laboratories, Hercules, CA).

Quantitative real-time PCR

Total RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA). To assess mRNA expression, quantitative real-time PCR was performed as described (23). The generated cDNA was amplified by using primers for human p21 (5'-ATGAGTTGGGAGGAGGCA-3' and 5'-AGAAGATGTGA-GAGCGGA-3'), p27 (5'-TGGAGAAACATCGACAGAC-3' and 5'-GCGTGTCTCAGAGTTAGCC-3') and glyceraldehyde 3-phosphate dehydrogenase (5'-GGTCTTACTCCTTG-GAGCCATGTG-3' and 5'-ACCTAACTACATGGTTTACAT-GTT-3').

Akt overexpression

Adenoviral vectors encoding enhanced GFP (EGFP) and hemagglutinin (HA)-tagged wild-type (WT) Akt (24) were amplified by infection of HEK293T cells as described (25). B16 cells were infected in serum-free medium for 1 h with 10 ml of HEK293T infected-cell supernatant, washed with PBS and transferred to 10% serum medium for 24 h to allow expression of the adenoviral genes. Cells were seeded and 24 h before cannabinoid treatment, cells were transferred to a medium containing 0.5% serum. Infection efficiency was controlled by counting fluorescent cells on control EGFP infections and was always higher than 80%. Expression of HA-tagged Akt was confirmed in the infected melanoma cells by Western blot analysis with antibodies against total Akt (see above) and HA (1:1000; Roche, Penzberg, Germany).

Statistics

Results shown represent mean ± SD. Statistical analysis was performed by ANOVA with a post hoc analysis by the Student-Newman-Keuls test or by unpaired Student's *t* test.

RESULTS

Melanoma cells express cannabinoid receptors

The expression of cannabinoid receptors in melanoma cells was examined by Western blot analysis, RT-PCR and confocal microscopy. Western blot experiments showed that CB₁ and CB₂ receptors were expressed in melanoma cell lines of murine and human origin (Fig.

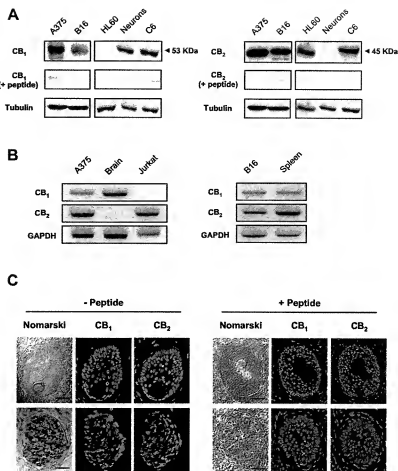


Figure 1. Melanoma cells express cannabinoid receptors. **A)** Western blot analysis. Human HL-60 promyelocytic cells, rat cortical neurons and rat C6 glioma cells were used as controls for CB₁ and/or CB₂ protein expression. Controls with anti-CB₁ and anti-CB₂ Ab blocking peptides are also shown. **B)** RT-PCR. Human brain-cortex extract (kindly given by María L. de Ceballos, Cajal Neuroscience Institute, Madrid, Spain), human Jurkat leukemia cells and mouse spleen were used as controls for CB₁ and/or CB₂ mRNA expression. **C)** Immunolocalization of CB₁ and CB₂ receptors (red) in two foci of a human cutaneous melanoma biopsy. Controls with anti-CB₁ and anti-CB₂ Ab-blocking peptides in two foci are also shown. Nuclei are stained in green. Scale bar: 100 μ m.

1A). The specificity of the anticannabinoid receptor antibodies used in the blotting experiments was assessed by antigen preabsorption with the corresponding blocking peptides. Expression of cannabinoid receptor mRNA was shown by RT-PCR (Fig. 1B). Moreover, immunostaining for CB₁ and CB₂ receptors was also analyzed by confocal microscopy in a series of human cutaneous melanoma biopsies. Of the 61 tumors examined, 36 expressed significant immunoreactivity for CB₁ and CB₂ receptors, 10 just for the CB₁ receptor, 10 just for the CB₂ receptor, and only 5 were negative for the two CB receptor types. An example of CB receptor-positive tumor is shown in Fig. 1C. As in the blotting experiments, the specificity of the anticannabinoid receptor antibodies used for immunostaining was assessed by antigen preabsorption (Fig. 1C).

Cannabinoids inhibit the growth of melanoma cells but not of normal melanocytes

We tested the functionality of cannabinoid receptors in the control of melanoma cell growth by using the plant-derived cannabinoid agonist THC and the synthetic cannabinoid agonist WIN-55,212-2. Both ligands bind to CB₁ and CB₂ receptors with comparable relative affinities and are therefore considered as mixed CB₁/CB₂ agonists [reported K_i values (nM) for CB₁ and CB₂

receptors, respectively: THC = 35–80 and 4–75; WIN-55,212-2 = 2–123 and 1–16 (reviewed in Ref. 8)]. THC and WIN-55,212-2 decreased the number of viable melanoma cells in the cultures (Fig. 2A, B). These effects were dose-dependent, and for example, values of cell density (as a percentage of the corresponding vehicle incubations) after 48 h of cannabinoid challenge were 71 ± 6 (1 μ M THC), 61 ± 6 (2 μ M THC), 39 ± 4 (2.5 μ M THC) and 22 ± 7 (3 μ M THC) for A375 cells. The growth of MelJuso cells, another human melanoma cell line, was also inhibited by cannabinoids (percentage of viability from the corresponding vehicle-treated cells after 48-h incubation: 74 ± 3 for 100 nM WIN-55,212-2 and 70 ± 2 for 1 μ M THC; $n=4$; cannabinoids vs. vehicle: $P<0.01$). Cannabinoid antiproliferative action on melanoma cells relied on CB receptor activation as the CB₁ antagonist SR141716 and the CB₂ antagonists SR144528 and AM630 prevented the effect of WIN-55,212-2 in B16 and A375 cells (Fig. 2A, B). In addition, cannabinoid antiproliferative action seemed to be selective for tumor cells, as WIN-55,212-2 and THC did not induce a statistically significant change in the number of viable mouse melan-c (Fig. 2C) and human Hermes 2b (Fig. 2D) cells. These two nontumorigenic lines of melanocytes expressed CB₁ receptors (Fig. 2C, D) at levels comparable to the

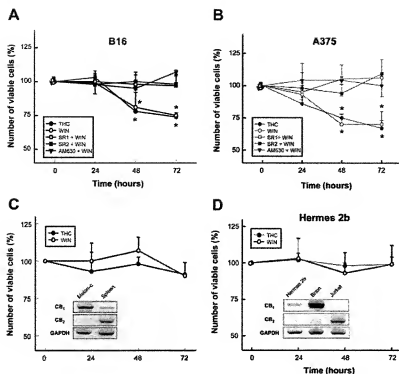


Figure 2. Cannabinoids inhibit the growth of melanoma cells but not of normal melanocytes in culture. *A, B*) The melanoma cell lines B16 (*A*) and A375 (*B*) were cultured for the times indicated with vehicle, 1 μ M THC or 100 nM WIN-55,212-2, either alone or in combination with 0.5 μ M SR141716 (SR1), 0.5 μ M SR144528 (SR2), or 1.0 μ M AM630, and the number of viable cells relative to vehicle incubations was determined ($n=5$). *Significantly different ($P<0.01$) from vehicle incubations. *C, D*) The nontransformed melanocytic lines melan-c (*C*) and Hermes 2b (*D*) were cultured for the times indicated with vehicle, 1 μ M THC or 100 nM WIN-55,212-2, and cell viability was determined ($n=4$). The inset shows the RT-PCR analysis of CB₁ and CB₂ mRNA expression in the respective cell lines.

tumorigenic A375 and B16 cell lines as determined by Western blot and RT-PCR (data not shown).

Cannabinoid administration inhibits melanoma progression and metastatic spreading in mice

Given the inhibition of melanoma cell growth by cannabinoids *in vitro*, we evaluated the effect of cannabi-

noid treatment *in vivo*. Tumors generated by inoculation of the highly malignant B16 cell line were treated with vehicle or WIN-55,212-2. As shown in **Fig. 3A**, cannabinoid administration notably reduced the growth of tumor cells *in vivo*. This antitumoral action of WIN-55,212-2 was studied in further detail.

1) Because cannabinoid-based therapeutic strategies should be as devoid as possible of psychotropic effects,

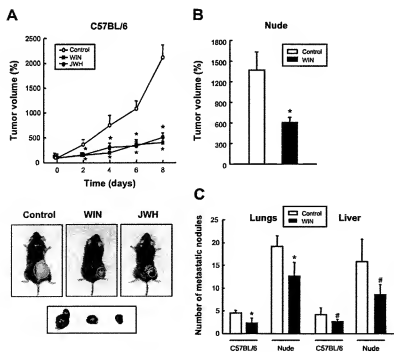


Figure 3. Cannabinoid administration inhibits melanoma progression and metastatic spreading in mice. *A*) Tumors were generated by s.c. injection of B16 cells in C57BL/6 mice, and animals were treated with either vehicle or cannabinoid (either WIN-55,212-2 or JWH-133 at 50 μ g/day, daily) for up to 8 days ($n=8$ for each experimental group). Tumor size was monitored during the treatment. Examples of tumors in the flank of mice and after dissection are shown. *Significantly different ($P<0.01$) from vehicle administration. *B*) Tumors were generated by s.c. injection of B16 cells in nude mice, and animals were treated with either vehicle or WIN-55,212-2 (50 μ g/day, daily) for 8 days ($n=6$ for each experimental group). *Significantly different ($P<0.01$) from vehicle administration. *C*) B16 cells were injected intrapleurally in C57BL/6 and nude mice, and animals were treated with either vehicle or WIN-55,212-2 (50 μ g/day, every 3 days) for 21 days (C57BL/6 mice, $n=6$) or 12 days (nude mice, $n=5$). The number of metastatic nodules in the lungs and the liver were subsequently counted. Significantly different (* $P<0.05$, ** $P<0.01$) from vehicle administration.

which are mediated by brain CB₁ receptors (9), and melanoma cells express CB₂ receptors (see above), which lack cannabinoid psychoactivity, we administered to mice JWH-133, a nonpsychoactive, CB₂-selective agonist. As shown in Fig. 3A, JWH-133 was as effective as the mixed CB₁/CB₂ agonist WIN-55,212-2 in preventing tumor growth.

2) To distinguish between direct WIN-55,212-2 antitumoral action on melanoma cells and potential immune-related responses induced by cannabinoid treatment, parallel experiments were conducted in immune-deficient (nude) mice. As shown in Fig. 3B, WIN-55,212-2 significantly inhibited melanoma growth in these animals.

3) To test whether the antitumoral effect of WIN-55,212-2 also targets melanoma cell spreading, we examined cannabinoid action in a model of metastatic-nodule formation. As shown in Fig. 3C, WIN-55,212-2 decreased the number of lung and liver metastases after systemic inoculation of melanoma cells to C57BL/6 and nude mice.

We next tested whether, as occurs in cultured melanoma cells, cannabinoids inhibit proliferation of tumor cells *in vivo*. Quantification of proliferative (=bromodeoxyuridine (BrdU)-labeled) cells in tumor sections revealed that treatment with WIN-55,212-2 or JWH-133

decreased tumor cell proliferation (Fig. 4A). This was accompanied by an increase in the number of apoptotic cells, as determined by TUNEL staining (Fig. 4B), and by a decrease in tumor vascularization, as determined by CD31 immunostaining (Fig. 4C). Morphometric analysis of the vasculature showed that the latter effect was associated with a decrease in vascular density (number of blood vessels per area unit: vehicle, 1.16 ± 0.10 ; WIN-55,212-2, 0.44 ± 0.08 ; JWH-133, 0.48 ± 0.07 ; $n=4$; cannabinoids vs. vehicle: $P<0.01$) and not to changes in vessel size (relative blood vessel sectional area: vehicle, 100 ± 16 ; WIN-55,212-2, 112 ± 36 ; JWH-133, 110 ± 35 ; $n=4$).

Cannabinoids inhibit melanoma cell cycle

The mechanism by which cannabinoids inhibit melanoma cell proliferation was investigated. Flow cytometry analysis showed that cannabinoid treatment inhibited B16 and A375 melanoma cell cycle, most likely at the G1-S transition, as shown by the increase of cells in the G0/G1 compartment and the decrease of cells in the S compartment (Fig. 5A, B). At the doses used in these experiments, no apoptotic effect of WIN-55,212-2 and THC was evident as inferred from the

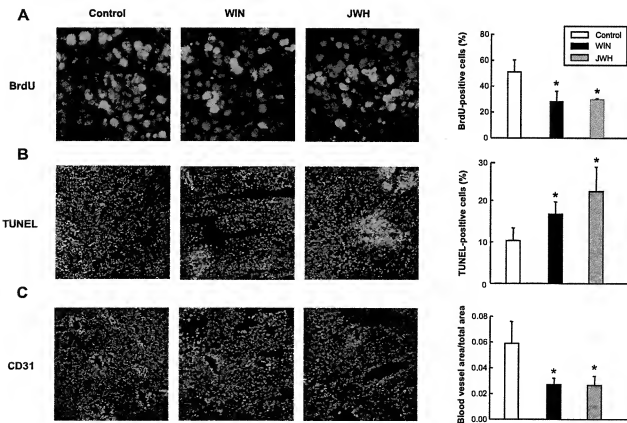


Figure 4. Effect of cannabinoid administration on melanoma cell proliferation, apoptosis, and angiogenesis in mice. Tumors were generated by s.c. injection of B16 cells in C57BL/6 mice, and animals were treated with either vehicle, WIN-55,212-2, or JWH-133 for 8 days. Five to ten fields of four to six sections from four different tumors were analyzed per group. Representative images are shown in each case. A) BrdU incorporation. B) TUNEL staining. C) CD31 immunostaining. *Significantly different ($P<0.01$) from vehicle administration.

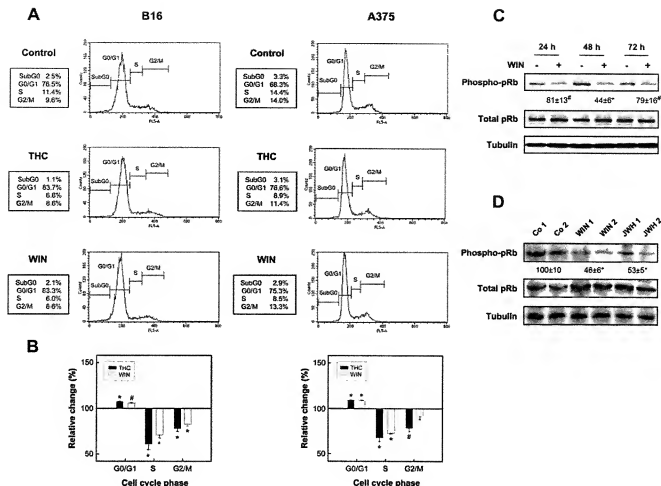


Figure 5. Cannabinoids inhibit melanoma cell cycle. **A, B)** Cells were cultured for 48 h with vehicle, 100 nM WIN-55,212-2, or 1 μ M THC, loaded with Hoechst 33342, and the cell cycle was analyzed by flow cytometry. Examples of cell cycle profiles are shown in **A**. The numbers indicate the percentage of cells at the different compartments. Quantification of the cannabinoid effect in 6 different experiments is shown in **B**. Significantly different ($^*P<0.05$, $^{**}P<0.01$) from vehicle incubations. **C)** B16 cells were cultured for the times indicated with vehicle or 100 nM WIN-55,212-2, extracts were obtained and Western blot analysis was performed. One representative experiment is shown. Optical density (OD) values (in percentage of the respective vehicle incubations; $n=4$) are given for phospho-pRb relative to total pRb. Significantly different ($^*P<0.05$, $^{**}P<0.01$) from vehicle incubations. **D)** Extracts were obtained from the tumors described in Fig. 4, and Western blot analysis was performed. Two representative tumors are shown for each condition. OD values ($n=4$) are given as in **C**. a Significantly different ($P<0.01$) from vehicle administration.

analysis of the sub-G0/G1 compartment. However, at higher concentrations ($\geq 2 \mu$ M for THC, $\geq 0.5 \mu$ M for WIN-55-212-2), both cannabinoids were also able to induce apoptosis as determined by 1) appearance of a hypodiploid (sub-G0/G1) population, 2) TUNEL staining, and 3) caspase 3 activation (data not shown).

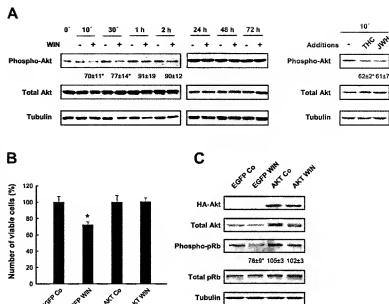
We next analyzed the levels of various proteins that are involved in the control of the cell cycle at the G1-S transition. In line with the aforementioned flow cytometry data, cannabinoids decreased the phosphorylation state of the retinoblastoma protein family member pRb, a master regulator of the G1-S transition (26), both in cultured melanoma cells (Fig. 5C) and in tumor xenografts (Fig. 5D). In contrast, no significant effect of cannabinoid challenge was observed on the levels of other classical members of the G1-S transition machinery, such as the cyclin-dependent kinases CDK2/3 and

CDK4; the CDK inhibitors p21 (WAF1; both protein and mRNA), and p27 (KIP1; both phosphorylated and total protein as well as mRNA); and the CDK4/6 activator cyclin D1 (Supplemental Fig. 1). In addition, the levels of total p53 and phospho-Ser-15-p53 were unaffected by cannabinoid treatment (Supplemental Fig. 1).

Akt is involved in cannabinoid-induced inhibition of melanoma cell proliferation

Cannabinoid receptors are known to modulate several pathways that are directly involved in the control of cell proliferation and survival, including phosphatidylinositol 3-kinase/Akt (27) and the extracellular signal-regulated kinase (ERK) (28), c-Jun N-terminal kinase (c-Jun

Figure 6. Akt is involved in cannabinoid-induced inhibition of melanoma cell proliferation. **A)** B16 cells were cultured for the times indicated with vehicle, 100 nM WIN-55,212-2, 1 μ M THC, or 100 nM JWH-133; extracts were obtained, and Western blot analysis was performed. One representative experiment is shown. OD values (in percentage of the respective vehicle incubations; $n=4$) are given for phospho-Akt relative to total Akt in short-term incubations. *Significantly different ($P<0.01$) from vehicle incubations. **B, C)** B16 cells were infected with HA-Akt or empty vector and further cultured for 48 h with vehicle or 100 nM WIN-55,212-2. The number of viable cells (**B**) and pRb phosphorylation status (**C**) were determined. Western blot controls of Akt expression in the different infection conditions and OD values for pRb are shown. Results correspond to four different experiments. *Significantly different ($P<0.01$) from vehicle incubations.



NH2-terminal kinase) (29, 30), and p38 mitogen-activated protein kinase (MAPK) (29, 30) cascades. We, therefore, examined the possible contribution of these pathways to the inhibition of B16 melanoma cell proliferation. Cannabinoid incubation induced a rapid inhibition of the prosurvival protein Akt (Fig. 6A), whereas ERK, JNK and p38 MAPK were not significantly affected (Supplemental Fig. 2).

To test the involvement of Akt in the growth-inhibiting effect of cannabinoids, we overexpressed Akt in B16 melanoma cells. We used an adenoviral vector to ensure that nearly all of the cells expressed the exogenous protein. Akt overexpression abrogated the antiproliferative effect of WIN-55,212-2 (Fig. 6B), as well as cannabinoid-induced pRb hypophosphorylation (Fig. 6C), suggesting that Akt inhibition is necessary for cannabinoid action.

DISCUSSION

Melanoma remains a management challenge. Despite many years of intensive research, currently approved therapies—high-dose IFN α -2b and dacarbazine—are only palliative or even ineffective, and ongoing therapeutic approaches, such as vaccine-based immunotherapy and targeted chemotherapy are as yet far from the clinics (2, 4, 5). Here, we studied the potential efficacy of cannabinoids as antitumoral agents against melanoma and show that these compounds exert a remarkable growth-inhibiting effect on melanoma cells *in vivo* that is evident under various experimental settings (animals with different immune statuses, melanoma cells inoculated at different sites, cannabinoids injected by different routes). Moreover, this is associated with an improvement of various tumor-progression parameters (decreased proliferation and vascularization, increased


apoptosis), as well as with an inhibition of tumor-cell metastatic spreading, one of the clinical hallmarks of advanced melanoma. In addition, cannabinoid action—at least *in vitro*—was selective for tumor *vs.* non-tumor cells, in line with previous studies on cells of astroglial (25, 31), epidermal (20), and thyroid-epithelial (32) origin, in which cannabinoids were able to kill tumor cells but not their nontransformed counterparts. Likewise, in primary astrocytes (25), oligodendrocytes (33) and mast cells (34), as well as in Chinese hamster ovary cells transfected with the CB₁ receptor cDNA (27), cannabinoids activate Akt, while in glioma cells they inhibit Akt (35). These and other findings support that cannabinoid receptors regulate cell survival pathways differently in tumor and nontumor cells and support the idea that cannabinoids are not expected to produce the generalized cytotoxic effects of conventional chemotherapies (11, 12).

Activation of cannabinoid receptors decreases melanoma cell proliferation, at least in part, via inhibition of Akt, a key element of a major prosurvival pathway that is deregulated in many types of tumors, including melanoma (5, 36, 37). Thus, Akt is constitutively activated in more than 60% of melanomas, with higher frequency of activation at later stages of the disease (2, 5). Several mechanisms may account for Akt overactivation in melanomas. For example, melanocytic lesions frequently show loss of PTEN, a lipid phosphatase that prevents Akt activation by phosphatidylinositol 3-kinase and which heterologous expression inhibits melanoma cell growth in culture and in nude mice (38). Loss of heterozygosity on regions of chromosome 10q, which harbors the PTEN locus, has been demonstrated in 30–50% of malignant melanomas, and ~10% of melanomas carry PTEN mutations (2, 38). Akt overactivation in melanomas may also be due to amplification of the AKT3 gene (39) and to mutations of the RAS gene

family, particularly NRAS, which appear in 10–20% of melanomas (40). Our findings also support that Akt inhibition arrests the cell cycle at the G1-S transition via pRb retinoblastoma protein hypophosphorylation. However, we cannot rule out that cannabinoid antiproliferative action in our system also relies on additional mechanisms.

The observed modulation of Akt but not of MAPK cascades in melanoma cells supports the pleiotropic coupling of cannabinoid receptors to different signaling pathways in different cell contexts. For example, it has been reported that cannabinoid receptors activate ERK (41) and Akt (25) in primary astrocytes, activate ERK (42) but do not affect Akt (27) in promyelocytic HL-60 cells, and activate ERK (14) and inhibit Akt (35) in C6 glioma cells. The precise reasons for these various signaling outcomes are as yet unclear, but as discussed elsewhere (11), it is conceivable that experimental factors such as the nature of the (endo)cannabinoid ligand (e.g., stability and hydrophobicity), the strength of signal input (e.g., agonist potency, dose and time of exposure), the biological properties of the target cell (e.g., origin, stage of differentiation and replicative capacity) and its cannabinoid receptors (e.g., number and G protein coupling), and the presence of various factors in the culture (e.g., growth factors and other cell types/clones) may affect cannabinoid receptor signaling properties.

Although the use of cannabinoids in medicine is limited by their psychotropic effects, these compounds display a fair drug safety profile; their potential adverse effects are within the range of those accepted for other medications—especially in cancer treatment—and their psychoactive effects tend to disappear with tolerance on continuous use (11, 12). Nonetheless, it is obvious that cannabinoid-based therapies devoid of side effects would be desirable. As the unwanted effects of cannabinoids are mediated largely or entirely by CB₁ receptors within the brain, the most conceivable possibility would be to use cannabinoids that selectively target CB₂ receptors. Although under certain circumstances CB₂ receptor activation in immune cells may blunt host antitumor activity, as discussed elsewhere (11, 43), this may be evident only in particular experimental conditions, and indeed most of the data obtained so far from animal models of cancer have demonstrated a tumor growth-inhibiting action of cannabinoids (11). In this context, here, we show that CB₂ receptor activation is functional in curbing melanoma cell growth *in vitro* and *in vivo*, the latter under conditions previously observed to produce no overt signs of psychoactivity (43). The apparent synergy between CB₁ and CB₂ receptors in inhibiting melanoma cell growth, as suggested by pharmacological antagonism experiments, is nonetheless intriguing, as it is not observed in the case of cannabinoid-induced apoptosis of glioma cells (14, 43). Of interest, nontransformed melanocytes only expressed CB₁ receptors, while melanoma cells expressed CB₁ and CB₂ receptors (cf. 44), in line with our previous observation the CB₂ receptor is expressed

in high-grade gliomas but not in primary astrocytes (43). Hence, the present report, together with the implication of CB₂ receptors in the control of processes such as pain initiation (45), emesis (46), and inflammation (47), opens the attractive possibility of finding cannabinoid-based therapeutic strategies devoid of undesired psychotropic side effects. Specifically, the antiproliferative effect of cannabinoids reported here may set the basis for a new therapeutic approach for the treatment of malignant melanoma. 

We are indebted to Christian Porres, Juan G. Fernández, Manuel Guerrero, Juan M. Blázquez, and Mónica García-Cosío for expert technical assistance, and to Joaquín Dopazo and Ismael Galve-Roperch for discussion and advice. This work was supported by grants from Fundación Científica de la Asociación Española Contra el Cáncer, Ministerio de Educación y Ciencia (SAF2003-00745), and Comunidad de Madrid-Universidad Complutense (950344) to M.G.; Ministerio de Educación y Ciencia (BMC2003-06098 and GEN2001-4856-C13-09), Comunidad de Madrid (GR/SAL/0223/2004), and Fundación Ramón Areces to M.M.; and Fondo de Investigaciones Sanitarias (RTICCC C03/10) to J.L.F.-L.

REFERENCES

- Edwards, B. K., Brown, M. L., Wingo, P. A., Howe, H. L., Ward, E., Ries, L. A. G., Schrag, D., Jamison, P. M., Jemal, A., Wu, X. C., et al. (2005) Annual report to the nation on the status of cancer, 1975–2002, featuring population-based trends in cancer treatment. *J. Natl. Cancer Inst.* 97, 1407–1427.
- Thompson, J. F., Scolyer, R. A., and Kefford, R. F. (2005) Cutaneous melanoma. *Lancet* 365, 687–701.
- National Cancer Institute, SEER Cancer Statistics Review 1975–2002: http://seer.cancer.gov/csr/1975_2002/results_merged/sect_16_melanoma.pdf (accessed June 1, 2006).
- Tsao, H., Atkins, M. B., and Sober, A. J. (2004) Management of cutaneous melanoma. *N. Engl. J. Med.* 351, 998–1012.
- Chudnovsky, Y., Khavari, F. A., and Adams, A. E. (2005) Melanoma genetics and the development of rational therapeutics. *J. Clin. Invest.* 115, 813–824.
- Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffon, G., Gibson, D., Mandelbaum, A., Etinger, A., and Mechoulam, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258, 1946–1949.
- Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., Gopher, A., Almog, S., Martin, B. R., Compton, D. R., et al. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* 50, 83–90.
- Howlett, A. C., Barth, F., Bonner, T. I., Cabral, G., Casellas, P., Devane, W. A., Felder, C. C., Herkenham, M., Mackie, K., Martin, B. R., Mechoulam, R., et al. (2002) International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol. Rev.* 54, 161–202.
- Piomelli, D. (2003) The molecular logic of endocannabinoid signalling. *Nat. Rev. Neurosci.* 4, 873–884.
- Tramer, M. R., Carroll, D., Campbell, F. A., Reynolds, D. J., Moore, R. A., and McQuay, H. J. (2001) Cannabinoids for control of chemotherapy induced nausea and vomiting: quantitative systematic review. *Br. Med. J.* 323, 16–21.
- Guzmán, M. (2003) Cannabinoids: potential anticancer agents. *Nat. Rev. Cancer* 3, 745–755.
- Hall, W., Christie, M., and Currow, D. (2005) Cannabinoids and cancer: causation, remediation, and palliation. *Lancet Oncol.* 6, 35–42.
- Munson, A. E., Harris, L. S., Friedman, M. A., Dewey, W. L., and Carchman, R. A. (1975) Antineoplastic activity of cannabinoids. *J. Natl. Cancer Inst.* 55, 597–602.

14. Galve-Roperch, I., Sánchez, C., Cortés, M. L., Gómez del Pulgar, T., Izquierdo, M., and Guzmán, M. (2000) Anti-tumoral action of cannabinoids: involvement of sustained ceramide accumulation and extracellular signal-regulated kinase activation. *Nat. Med.* 6, 313–319.
15. Bifulco, M., and Di Marzo, V. (2002) Targeting the endocannabinoid system in cancer therapy: a call for further research. *Nat. Med.* 8, 547–550.
16. Chan, P. C., Sills, R. C., Braun, A. G., Haseman, J. K., and Bucher, J. R. (1996) Toxicity and carcinogenicity of Δ^9 -tetrahydrocannabinol in Fischer rats and B6C3F1 mice. *Fund. Appl. Toxicol.* 30, 109–117.
17. Grotenhermen, F. (2003) Pharmacokinetics and pharmacodynamics of cannabinoids. *Clin. Pharmacokinet.* 42, 527–560.
18. Iversen, L. (2005) Long-term effects of exposure to cannabis. *Curr. Opin. Pharmacol.* 5, 69–72.
19. Guzmán, M., Duarte, M. J., Blázquez, C., Ravina, J., Rosa, M. C., Galve-Roperch, I., Sánchez, C., Velasco, G., and González-Feria, L. (2006) A pilot clinical study of Δ^9 -tetrahydrocannabinol in patients with recurrent glioblastoma multiforme. *Br. J. Cancer.* 95, 197–203.
20. Casanova, M. L., Blázquez, C., Martínez-Palacio, J., Villanueva, C., Fernández-Aceñero, M. J., Huffman, J. W., Jorcano, J. L., and Guzmán, M. (2003) Inhibition of skin tumor growth and angiogenesis *in vivo* by activation of cannabinoid receptors. *J. Clin. Invest.* 111, 43–50.
21. Blázquez, C., Casanova, M. L., Planas, A., Gómez del Pulgar, T., Villanueva, C., Fernández-Aceñero, M. J., Aragón, J., Huffman, J. W., Jorcano, J. L., and Guzmán, M. (2003) Inhibition of tumor angiogenesis by cannabinoids. *FASEB J.* 17, 529–531.
22. Blázquez, C., González-Feria, L., Álvarez, L., Haro, A., Casanova, M. L., and Guzmán, M. (2004) Cannabinoids inhibit the vascular endothelial growth factor pathway in gliomas. *Cancer Res.* 64, 5617–5623.
23. Gutiérrez, O., Pipson, C., Inohara, N., Fontalba, A., Ogura, Y., Prosper, F., Núñez, G., and Fernández-Luna, J. L. (2002) Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor- κ B activation. *J. Biol. Chem.* 277, 41701–41705.
24. Kitamura, T., Ogawa, W., Sakaue, H., Hino, Y., Kuroda, S., Takata, M., Matsumoto, M., Maeda, T., Konishi, H., Kikkawa, U., and Kasuga, M. (1998) Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. *Mol. Cell Biol.* 18, 3708–3717.
25. Gómez del Pulgar, T., de Ceballos, M. L., Guzmán, M., and Velasco, G. (2002) Cannabinoids protect astrocytes from ceramide-induced apoptosis through the phosphatidylinositol 3-kinase/protein kinase B pathway. *J. Biol. Chem.* 277, 36527–36533.
26. Malumbres, M., and Barbacid, M. (2001) To cycle or not to cycle: a critical decision in cancer. *Nat. Rev. Cancer.* 1, 222–231.
27. Gómez del Pulgar, T., Velasco, G., and Guzmán, M. (2000) The CB₂ cannabinoid receptor is coupled to the activation of protein kinase B/Akt. *Biochem. J.* 347, 369–373.
28. Bouaboula, M., Poinot-Chazel, C., Bourrié, B., Canat, X., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., and Casellas, P. (1995) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB₁. *Biochem. J.* 312, 637–641.
29. Liu, J., Gao, B., Mirshahi, F., Samal, A. J., Khanolkar, A. D., Makriyannis, A., and Kumos, G. (2000) Functional CB₁ cannabinoid receptors in human vascular endothelial cells. *Biochem. J.* 346, 835–840.
30. Rueda, D., Galve-Roperch, I., Haro, A., and Guzmán, M. (2000) The CB₁ cannabinoid receptor is coupled to the activation of c-Jun N-terminal kinase. *Mol. Pharmacol.* 58, 814–820.
31. McAllister, S., Chan, C., Taft, R., Lau, T., Abood, M., Moore, D., Aldape, K., Yount, G. (2005) Cannabinoids selectively inhibit proliferation and induce death of cultured human glioblastoma multiforme cells. *J. NeuroOncol.* 74, 31–40.
32. Bifulco, M., Iacocca, G., Portella, G., Vitale, M., Orlando, P., De Petrosellis, L., and Di Marzo, V. (2001) Control by the endogenous cannabinoid system of ras oncogene-dependent tumor growth. *FASEB J.* 15, 2745–2747.
33. Molina-Holgado, E., Vela, J. M., Arevalo-Martín, A., Almazán, G., Molina-Holgado, F., Borrell, J., and Guaza, C. (2002) Cannabinoids promote oligodendrocyte progenitor survival: involvement of cannabinoid receptors and phosphatidylinositol 3-kinase/Akt signaling. *J. Neurosci.* 22, 9742–9753.
34. Samson, M. T., Small-Howard, A., Shimoda, L. M., Kobian-Huberson, M., Stokes, A. J., and Turner, H. (2003) Differential roles of CB₁ and CB₂ cannabinoid receptors in mast cells. *J. Immunol.* 170, 4953–4962.
35. Gómez del Pulgar, T., Velasco, G., Sánchez, C., Haro, A., and Guzmán, M. (2002) De novo-synthesized ceramide is involved in cannabinoid-induced apoptosis. *Biochem. J.* 363, 183–188.
36. Bellacosa, A., Kumar, C. C., Di Cristofano, A., and Testa, J. R. (2005) Activation of AKT kinases in cancer: implications for therapeutic targeting. *Adv. Cancer Res.* 94, 29–86.
37. Chudnovsky, Y., Adams, A. E., Robbins, P. B., Lin, Q., and Khavari, P. A. (2005) Use of human tissue to assess the oncogenic activity of melanoma-associated mutations. *Nat. Genet.* 37, 745–749.
38. Wu, H., Goel, V., and Haluska, F. G. (2003) PTEN signaling pathways in melanoma. *Oncogene.* 22, 3113–3122.
39. Stahl, J. M., Sharma, A., Cheung, M., Zimmerman, M., Cheng, J. Q., Bosenberg, M. W., Kester, M., Sandrasegaran, L., and Robertson, G. P. (2004) Deregulated Akt5 activity promotes development of malignant melanoma. *Cancer Res.* 64, 7002–7010.
40. Chin, L. (2003) The genetics of malignant melanoma: lessons from mouse and man. *Nat. Rev. Cancer.* 3, 559–570.
41. Sánchez, C., Galve-Roperch, I., Rueda, D., and Guzmán, M. (1998) Involvement of sphingomyelin hydrolysis and the mitogen-activated protein kinase cascade in the Δ^9 -tetrahydrocannabinol-induced stimulation of glucose metabolism in primary astrocytes. *Mol. Pharmacol.* 54, 834–843.
42. Bouaboula, M., Poinot-Chazel, C., Marchand, J., Canat, X., Bourrie, B., Rinaldi-Carmona, M., Calandra, B., Le Fur, G., and Casellas, P. (1996) Signaling pathway associated with stimulation of CB₂ peripheral cannabinoid receptor. Involvement of both mitogen-activated protein kinase and induction of Krox-24 expression. *Eur. J. Biochem.* 237, 704–711.
43. Sánchez, C., de Ceballos, M. L., Gómez del Pulgar, T., Rueda, D., Corbacho, C., Velasco, G., Galve-Roperch, I., Huffman, J. W., Ramón y Cajal, S., and Guzmán, M. (2001) Inhibition of glioma growth *in vivo* by selective activation of the CB₂ cannabinoid receptor. *Cancer Res.* 61, 5784–5789.
44. Segal, N. H., Pavlidis, P., Noble, W. S., Antonescu, C. R., Viale, A., Wesley, U. V., Busam, K., Gallardo, H., DeSanis, D., Brennan, M. et al. (2003) Classification of clear-cell sarcoma as a subtype of melanoma by genomic profiling. *J. Clin. Oncol.* 21, 1775–1781.
45. Calignano, A., La Rana, G., Giuffrida, A., and Piomelli, D. (1998) Control of pain initiation by endogenous cannabinoids. *Nature* 394, 277–281.
46. Van Sickle, M. D., Duncan, M., Kingsley, P. J., Mouhate, A., Urbani, P., Mackie, K., Stella, N., Makriyannis, A., Piomelli, D., Davison, J. S., et al. (2005) Identification and functional characterization of brainstem cannabinoid CB₂ receptors. *Science* 310, 329–332.
47. Klein, T. W. (2005) Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nat. Rev. Immunol.* 5, 400–411.

Received for publication June 8, 2006

Accepted for publication July 31, 2006